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胀果甘草悬浮培养细胞合成甘草总黄酮

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摘要：比较了胀果甘草（*Glycyrrhiza inflata*）悬浮细胞在逐级放大摇瓶中的生长、黄酮产量以及营养消耗过程，以便了解其放大规律。结果表明，在 250 和 500 mL 摇瓶中，细胞的最大生物量、黄酮产量以及最大比生长速率没有显著性差异，但是在 1 L 的摇瓶中，这三种参数都显著地降低，分别比 250 mL 摇瓶中降低了 27%，30%和 27%。在逐级放大的摇瓶中，氮、磷、铵浓度都随着培养时间延长而逐渐降低，尽管在 1 L 的摇瓶中磷消耗得最慢，但三种摇瓶中磷在细胞生长对数期基本都被消耗尽了。此外，硝态氮在第 18 天时基本被消耗完，而铵态氮在细胞收获时仍能维持在 100 mg/L。因此在反应器中培养时，主要的培养条件还需进一步优化。

关键词：胀果甘草细胞；生长；黄酮产量；营养消耗；放大培养
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Production of Flavonoids in Cell Suspension Culture of *Glycyrrhiza inflata* (Leguminosae)

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Abstract: The cell biomass, flavonoids production and nutrients consumption were monitored to characterize the culture of progressive scale-up of *Glycyrrhiza inflata*. The maximum biomass, flavonoids production and the maximum specific growth rate in the culture of 250 and 500 mL flasks remain similar, but were significantly higher than that of 1 000 mL flask. The three parameters in the culture of 1 000 mL flask were 27%, 30%, and 27% lower than that of 250 mL flask, respectively. The concentrates of phosphate, nitrate and ammonium in progressive scale-up flasks decreased similarly. Phosphate and nitrate were almost exhausted on day 10 and 18 in all flasks, respectively; while ammonium maintained about 100 mg/L till cells were harvested. The basic culture conditions needed to be further optimized for higher flavonoids production on a bioreactor scale.

Key words: *Glycyrrhiza inflata*; Growth; Flavonoids; Nutrients consumption; Scale-up flask

Glycyrrhiza inflata Bat. is a traditional Chinese medicine. One of the major compounds, flavonoids have significant anti-oxidation activity, antitumor activity (Kanazawa *et al*. 2003), antihuman immunodeficiency virus activity (Hatano *et al*. 1988) and antibacterial activity (Fukai *et al*. 2002).

Thus, the *G. inflata* plant has fallen short of supply with an increasing demand for flavonoids. Plant cell culture is a useful method for the production of valuable secondary metabolites.

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However, there were few studies on cell suspension culture of *G. inflata*, and its nutrients consumption has not yet been systematically investigated . Cell growth and flavonoids biosynthesis are sensitive to environmental conditions . Furthermore, scaling up the culture from flask to bioreactor is very difficult . Consequently, in the present study, the cell growth, flavonoids accumulation and nutrient consumption were investigated in scale-up flasks to understand the characteristic of *G. inflata* cell, which may be beneficial for scaling up in the bioreactor .

Materials and Methods

G. inflata cell were maintained in Murashige and Skoog (MS) culture medium containing 3% sucrose supplemented with 2,4-dichlorophen oxyacetic acid (2,4-D, 0.5mg/L), naphthalene acetic acid (NAA, 0.5mg/L) and 6-benzyladenine (6-BA, 0.5mg/L) . For experiments, cells were cultured at 25℃ on a rotatory shaker (120 r/min) with 5% inoculum in 250, 500 mL and 1 L flasks with 70, 140 and 280 mL medium (pH 5.8), respectively . The cells were harvested by filtration, and dried at 50℃ to constant dry weight (DW) . The maximum specific growth rates were calculated by the formula: $(X_2 - X_1) / (t_2 - t_1)$, where X_2 and X_1 were the DW of the cell at t_2 and t_1 days during the exponential growth phase, respectively .

Nitrate in the medium was determined according to Hecht & Mohr (1990) . Salicylic acid-H_2SO_4 (5%) was put into the medium, 20 min later, 8% NaOH was added in . Then the absorbency was determined at 410 nm at 25℃ . Ammonium was assayed as reported by Moore & Stein (1948) . After 2 mL dilute medium, 3 mL ninhydrin reagent and 0.1 mL 1% ascorbic acid were incubated together in 100℃ water for 15 min, and slowly cooled in air . The absorbency was determined at 580 nm . Additionally, phosphate was determined by colorimetry at 660 nm when 1 mL dilute medium and 3 mL phosphate reagent (2.5% ammonium molybdate∶10% ascorbic acid∶water = 1∶1∶2) was placed in 45℃ water for 20 min (Li, 1998) .

The flavonoids were extracted with 30 volumes of ethanol/water (70/30, v/v) by sonication for 1 h at 25℃ . After centrifugation at 10 000 rpm for 6 min, the supernatant was extracted three times with EtOAc, then with 95% ethanol . The combination of flavonoids in cells and medium was determined by colorimetry according to Zhang *et al* . (2001) . Rutin was used as a standard sample .

Results and Discussion

Cultured with a cycle of 22 days, the maximum biomass, flavonoids production and the maximum specific growth rate of the cells all decreased with progressive scale-up flasks, and by 27%, 30% and 27% in 1 L flasks compared to that in 250 mL flasks, respectively (Table 1) . But the differences were insignificant between 250 and 500 mL flasks ($P > 0.05$), which was agreed with the observation in *Catharanthus roseus* cell suspension culture (Zheng *et al* . 1998) . Additionally, a little foam was observed on the surface of the

Table 1 Compare of the maximum biomass, flavonoids production and maximum specific growth rate in cell suspension culture of *G. inflata* in progressive scale-up flasks . Data with different superscript letters are significantly different (one-way ANOVA followed by a multiple comparison; a, b: $P < 0.05$; B: $P < 0.01$, n = 3) .

Flasks	Maximum biomass (g DW/L)	Flavonoids production (mg/L)	Maximum specific growth rate (g DW/L/d)
250 mL	14.5 ± 0.61^{a}	80.35 ± 4.2^{a}	0.66 ± 0.03^{a}
500 mL	13.3 ± 0.82^{a}	73.63 ± 4.1^{a}	0.61 ± 0.04^{a}
1 L	10.6 ± 0.75^{B}	56.45 ± 1.4^{B}	0.48 ± 0.04^{b}

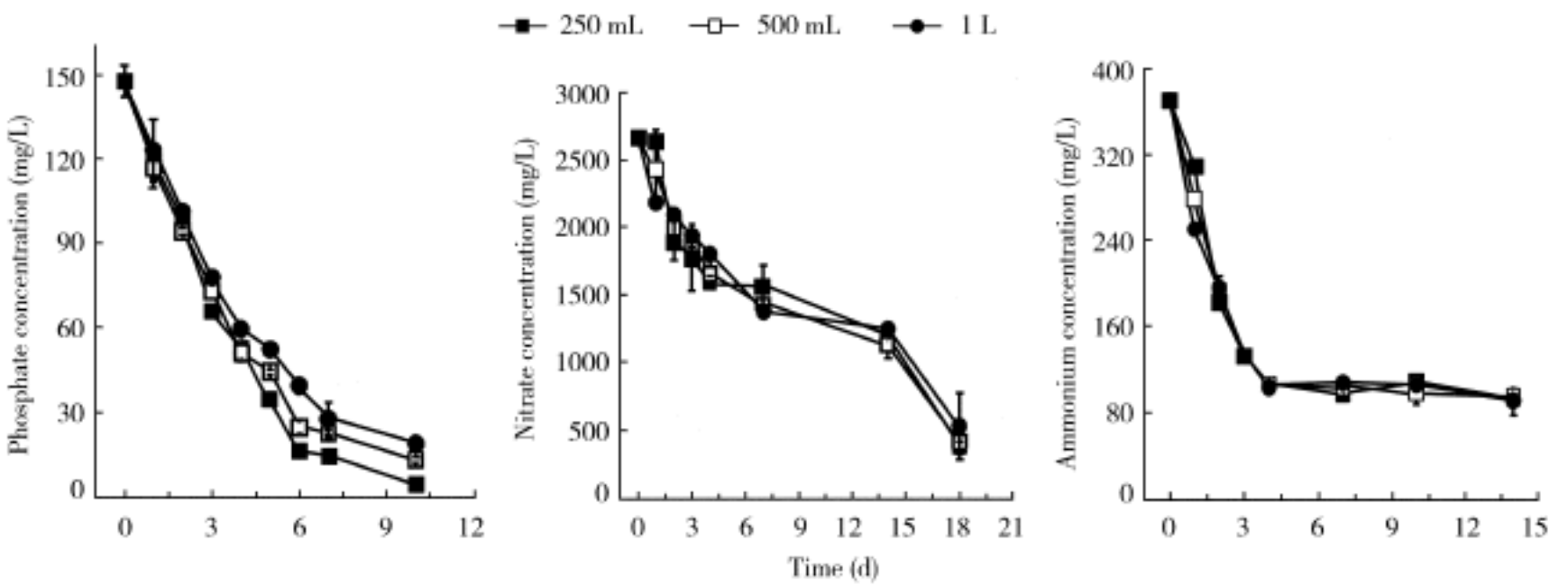

Fig . 1 Time courses of phosphate, nitrate and ammonium consumption in cell suspension culture of *G. inflata* grown in MS medium ■, □ and ● represent for 250, 500 mL and 1 L flasks, respectively . Each data indicated the means of three independent experiments .

1 L flasks in which the cells appeared brown and less healthy in the later stage of cultivation . Zheng *et al* . (1998) found that the oxygen mass transfer ($K_L a$) in 10 L bioreactor was significantly higher than that in flasks . Ten Hoopen (1994) reported that shear stress was the main reason making the cells brown . Thus, the decreased biomass of brown cell and flavonoids production in 1 L flasks might be due to shear stress and the decreased dissolved oxygen .

The trends of phosphate, nitrate and ammonium consumption in progressive scale-up flasks were very similar that the nutrients all decreased sharply (Fig . 1) . At the initial several days, the phosphate consumptions in 250, 500 mL and 1 L flasks were almost alike, but later it was the slowest in 1 L flasks . The phosphate in all flasks was almost exhausted on day 10 at the logarithm phase, which was observed in many plants cell cultures (Shin *et al* . 2003) . Most plant cells utilize ammonium firstly and nitrate later (Shin *et al* . 2003) . In the study, the ammonium consumption was faster than nitrate at the beginning of the cultivation . Low concentrations of ammonium stimulate nitrate uptake and of nitrate inhibite ammonium uptake (Dortch, 1990) . Thus, the nitrate was almost run out of on day 18, while the ammonium still maintained about 100 mg/L when the cells were harvested . Consequently, the main culture conditions needed to be further optimized for higher flavonoids production on a bioreactor scale .

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